Chronic Exposure to Ethanol Alters GABA\textsubscript{A} Receptor-Mediated Responses of Layer II Pyramidal Cells in Adult Rat Piriform Cortex

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**Signore, Armando P. and Hermes H. Yeh.** Chronic exposure to ethanol alters GABA\textsubscript{A} receptor-mediated responses of layer II pyramidal cells in adult rat piriform cortex. *J Neurophysiol* 84: 247–254, 2000. This study examined the effect of chronic exposure to ethanol on \(\gamma\)-aminobutyric acid type-A (GABA\textsubscript{A}) receptor-mediated responses of layer II pyramidal neurons of the piriform cortex. Slices containing the piriform cortex were derived from pair-fed adult rats maintained on ethanol-supplemented or control liquid diet for 30 days. Responses of identified layer II pyramidal neurons to exogenously applied GABA were monitored by whole-cell patch-clamp recording. Chronic exposure to ethanol resulted in a rightward shift in the EC\textsubscript{50} of GABA and a decrease in the amplitude of maximal GABA response. GABA-induced responses were modulated by acutely applied ethanol (10–100 mM) in both chronic ethanol-treated and control groups. No significant difference was found in the average change in GABA response, suggesting that tolerance to acute ethanol exposure did not develop. When the modulatory responses of individual cells were classified and grouped as either being attenuating, potentiating, or having no effect, the incidence of potentiation in the ethanol-treated group was significantly higher. Consistent with the absence of tolerance to acute ethanol, cross-tolerance to diazepam was not observed following 30 days of treatment with ethanol. These results are discussed in light of regionally specific effects of chronic ethanol treatment on GABA\textsubscript{A} receptor-mediated responses of layer II piriform cortical neurons.

**INTRODUCTION**

Several classes of neurotransmitter receptors, among them the inhibitory ionotropic \(\gamma\)-aminobutyric acid type-A (GABA\textsubscript{A}) receptor, are implicated in contributing to the acute intoxicating effects and long-term deleterious influences of ethanol on the CNS (Deitrich et al. 1989; Grant 1995; Grobin et al. 1998; Littleton and Little 1994; Nevo and Hamon 1995; Samson and Harris 1992; Sytinsky et al. 1975). Indeed, there is considerable evidence that both acute and chronic exposure to ethanol alters the function of GABA\textsubscript{A} receptors (Allan and Harris 1987; Macdonald and Olsen 1994; Sanna et al. 1993). Nonetheless, in either the acute or the chronic condition, the mechanisms underlying the effects of ethanol on the GABA\textsubscript{A} receptor remain to be elucidated.

Depending on whether the exposure is acute or chronic, ethanol appears to modulate the functional properties of GABA\textsubscript{A} receptors with variable outcomes. In the majority of cases, acute exposure to ethanol results in enhanced GABA\textsubscript{A} receptor function (Chandler et al. 1998; Nestoros 1980; Nishio and Narahashi 1990; Reynolds and Prasad 1991; Soldo et al. 1998; Suzdek et al. 1986). Chronic exposure to ethanol, on the other hand, decreases GABA\textsubscript{A} receptor function, consistent with the development of tolerance to the acute effects of ethanol (Allan and Harris 1987; Rastogi et al. 1986; Ticku 1980). In addition, an apparent cross-tolerance develops consequent to chronic ethanol exposure since there is a demonstrable concomitant loss of the allosteric potentiating effects of certain GABA\textsubscript{A} receptor modulators, such as benzodiazepines (Buck and Harris 1990) or barbiturates (Morrow et al. 1988). Cross-tolerance to GABA\textsubscript{A} receptor modulators is seen in human alcoholism as well as in animal models in which the effects of chronic ethanol exposure have been tested (Miller 1995).

Factors such as experimental preparation (Sapp and Yeh 1998), regional selectivity (Givens and Breese 1990; Matthews et al. 1998; Soldo et al. 1994), and developmental age (Lu and Yeh 1999) are important considerations in investigating the effects of ethanol on the CNS. For example, considerable research has been done using dissociated preparations, such as microsacs and brain homogenates (Allan and Harris 1987; Buck and Harris 1990; Morrow et al. 1988; Rastogi et al. 1986; Sanna et al. 1990, 1993). These methodologies, while effective in delineating the essential effect of ethanol on native GABA\textsubscript{A} receptors, do not distinguish between different cell populations within a given brain region or even among brain regions, thus compromising the ability to discern cell type- or region-specific effects of ethanol. Dissociated cells, tissue culture, and brain slices facilitate the analysis of specific cell types (Tatebayashi et al. 1998), but have typically relied on tissue derived from neonatal or young postnatal animals. Overall, it has been difficult to obtain an adult brain slice preparation in which neurons remain viable for patch-clamp electrophysiological analysis of GABA\textsubscript{A} receptor function (Thibault et al. 1995). In this light, information on the effects of chronic ethanol on anatomically intact, live adult CNS neurons is relatively scant (Freund et al. 1993; Nestoros 1980; Palmer and Hoffer 1990) and has been limited to studies employing intra- and extracellular recording approaches in vivo (Molleman and Little 1995;...

To address issues related to the effects of chronic ethanol on the adult brain, we developed an adult brain slice preparation and obtained viable brain slices from rats that have been subjected to an established chronic ethanol liquid-diet regimen (Liebar and DeCarli 1982). In the present study, we focused on the piriform cortex, a region of the brain that subserves olfaction and is affected by chronic use of ethanol in humans. Indeed, deficits in olfaction are well-documented in alcoholics (Ditraglia et al. 1991; Gregson et al. 1981; Kessler et al. 1991; Shear et al. 1992; Squires et al. 1985). In addition, plastic changes leading to degeneration of the piriform cortex can be induced in rats by repeated exposure to ethanol (Collins et al. 1996, 1998; Corso et al. 1998; Crews et al. 1999; Switzer et al. 1981). In this study, we postulated that one of the plastic changes might involve a change in the sensitivity of piriform cortical neurons to GABA. The responses of pyramidal cells in layer II of the rat piriform cortex to GABA were therefore examined by whole-cell patch-clamp recording, and GABA concentration-response relationships were compared between rats that were chronically treated with ethanol and pair-fed control animals. The sensitivity of GABA-mediated current responses in piriform pyramidal neurons to modulation by acutely applied ethanol and diazepam was also examined. We found that in adult rats chronically exposed to ethanol, pyramidal cells in layer II of the piriform cortex were less sensitive to GABA, but that sensitivity to modulation by diazepam remained unaltered. Concomitantly, in the chronic ethanol-treated group, there was an increase in the incidence of pyramidal neurons with GABA-induced responses that were potentiated by acute exposure to ethanol.

METHODS

Chronic treatment with ethanol

Adult male Sprague-Dawley rats (200–280 g at start of study) were pair-fed for 30 days with either a liquid diet (Research Diets, Inc., New Brunswick, NJ) supplemented with ethanol (5% wt/vol; “chronic ethanol,” n = 12) or an isocaloric control diet containing maltose-dextrin (“control,” n = 14) (Lieber and DeCarli 1982; Vavrauské-Jakubí et al. 1991; Wiener et al. 1981). The liquid diets were prepared fresh each day and were available ad libitum along with water. Following this regimen, rats in the chronic ethanol-fed group tended to gain weight at a slower rate relative to the control group. To minimize the weight differential at the end of the 30-day liquid-diet regimen (377 ± 12 g in the control group versus 332 ± 6 g in the chronic ethanol group), rats were weighed daily and the amount of food given to each control rat was adjusted to match the intake of the ethanol-fed rats for the entire 30-day treatment period.

Preparation of adult piriform cortical slices

Rats were killed and perfused transcardially with ice-cold, low-chloride artificial cerebral spinal fluid (low-chloride aCSF) containing (in mM): 140 sodium isethionate, 2.0 KCl, 4.0 MgCl₂, 0.1 CaCl₂, 25 NaHCO₃, 25 glucose. Brains were quickly removed, bisected along the sagittal plane, immersed in ice-cold low-chloride aCSF, and 250–400 μm coronal slices containing the anterior piriform cortex were cut using a vibriscaler (Campden Instruments, Shelley, UK). Slices were then stored at room temperature in Dulbecco’s modified Eagle’s medium with high glucose (25 mM) and bicarbonate (25 mM) and used for electrophysiological recording within 6 h of cutting. All solutions used for cutting and incubating slices were bubbled continuously with 5% CO₂/95% O₂.

Electrophysiology

Adult piriform cortical slices were placed in a custom-made recording chamber, stabilized by an overlying platinum ring strut with a plastic mesh, and perfused with normal artificial cerebral spinal fluid (aCSF) containing (in mM): 124 NaCl, 5.0 KCl, 2.0 MgCl₂, 2.0 CaCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, 10 glucose. The rate of perfusion was approximately 0.5 ml/min. Slices were viewed under Hoffman Modulation Contrast optics (Modulation Optics, Greenvale, NY) using a 40× extra-long working distance water immersion lens mounted on a fixed-stage upright microscope (Olympus, Woodbury, NY). A charge-coupled device (CCD) camera (Dage MTI, Michigan City, IN) was also used to view and collect images of the cells examined. Digitized images were collected and stored on a PC clone using image capture software (Flashpoint Integral Technologies, Indianapolis, IN).

Patch-clamp recording in the whole-cell configuration (Hamill et al. 1981) was employed to assess GABA A receptor-mediated activity. The recording electrodes were pulled from fiber-filled borosilicate glass capillary tubes (Sutter Instruments, Novato, CA) to an input resistance of 6–8 MΩ when filled with a recording solution containing (in mM): 140 KCl, 1.0 MgCl₂, 1.8 CaCl₂, 3 Mg-ATP, 119.15 K-HEPES, pH 7.3 with KOH. The lidocaine derivative QX-222 (RBI, Natick, MA) was included at a concentration of 5 mM to eliminate sodium channel activity. Lucifer yellow (0.5%, Molecular Probes, Eugene, OR) was included to facilitate visualization of the cells. During whole-cell recording, the Lucifer yellow readily filled the soma and processes of the cell under study; this was revealed by epi-illumination using an Olympus U-MWIB filter (Fig. 1C).

Seal formation and recordings were conducted using an EPC-7 amplifier (Darmstadt, Germany). Cells were voltage clamped at −70 mV and the recording was performed at room temperature. Liquid junction potentials were nulled prior to the start of each recording. Membrane currents were amplified and filtered through a four-pole Bessel filter. The analog signals were monitored on-line using a chart recorder (Gould, Valley View, OH). Digitized data were also acquired (DATAQ, JPM Programming) and stored for off-line analysis.

Peak amplitude of GABA-induced current responses were determined using Igor Pro (WaveMetrics, Inc., Lake Oswego, OR). From this data set, GABA concentration-response relationships and estimations of EC₅₀ were made using Sigma Plot version 5.0 (SPSS, Chicago, IL). The effect of ethanol or diazepam on GABA-induced responses were quantified by comparing the peak amplitude of GABA responses averaged from a set of at least three identical and consecutive trials each taken before (control), during, and after exposure to ethanol or diazepam. The percentage change in the amplitude of GABA response from the control response was calculated as

$$ \text{Percent change} = \left( \frac{I_{\text{max}} - I_{\text{control}}}{I_{\text{control}}} \right) \times 100 $$

where $I_{\text{max}}$ represents the mean peak amplitude of GABA-induced current during exposure to ethanol or diazepam, and $I_{\text{control}}$ represents the mean peak amplitude of GABA-induced current during the control period. Statistical analysis was performed using Statmost version 3.2 for Windows 95 (Datafax, Los Angeles, CA). Analysis of covariance was calculated by using a pooled fit method and the F-distribution (Motulsky and Ransnas 1987). Data were reported as mean ± SE.

Focal applications of drugs

GABA, ethanol, and diazepam were dissolved in aCSF and loaded into separate barrels of an eight-barrel glass pipette assembly. The multibarrel assembly was pulled to a fine point, filled, and the tip was broken under microscopic control such that the outer diameter of each
barrel was approximately 1.5 μM. Drug solutions were ejected by regulated pressure (≈3 psi) and, as shown in Fig. 1B (double arrowheads), were delivered within 10 μm of the cell under study. The application of drugs was routinely directed to the basal portion of the soma. One of the barrels of the multibarrel assembly was routinely filled with ACSF, which was applied to clear drugs from the vicinity of the cell and to control for mechanical artifacts due to bulk flow.

For assessing the concentration-response relationship, the maximal amplitude of the GABA-induced response was obtained by a 2- or 3-s pressure application of GABA at varying concentrations (1–200 μM). In experiments involving tests of interaction between GABA and ethanol or diazepam, GABA was applied at equal intervals (10–30 s) at a concentration of 10 μM (approximately EC25 of the GABA response) for 100 ms to 1 s. Ethanol (10–100 mM) was prepared fresh immediately prior to each recording session. Diazepam was first dissolved in DMSO and stored frozen as stock at a concentration of 10 μM. Bicuculline methiodide was stored as 200 mM stock and diluted to 50 μM. Unless otherwise indicated, all drugs and chemicals were purchased from Sigma (St. Louis, MO).

RESULTS

Identity of cells in piriform cortex

Figure 1A illustrates a coronal slice containing piriform cortex obtained from the adult rat brain. Pyramidal cells from layer II were identified in these slices. One such cell typically selected for patch-clamp recording is shown in Fig. 1B. This cell appeared phase bright and emitted a primary apical dendrite that was directed toward the lateral olfactory tract. Intracellular diffusion of Lucifer yellow included in the whole-cell recording solution allowed visualization of the neuronal cell body and the dendritic arbor under epi-illumination (Fig. 1C).

Large layer II cells displaying an apical dendrite and not more than two basal dendrites were classified as pyramidal cells. Those that did not fit these criteria were rejected from the data pool.

GABA concentration-response relationship

Application of GABA elicited robust, reversible, and concentration-dependent inward currents in pyramidal cells held at −70 mV. The penwriter record in the inset of Fig. 2A illustrates an experiment to determine the GABA concentration-response relationship in a pyramidal cell recorded in a control slice. In both control and chronic ethanol-treated groups, pyramidal cells displayed a threshold for response to GABA at approximately 3 μM, while GABA delivered at ≥60 μM resulted in a saturated maximal current response. The GABA-induced current was blocked by 50 μM bicuculline methiodide, a competitive antagonist of the GABA_A receptor (Fig. 2B).

To examine whether exposure to chronic ethanol shifted the sensitivity to GABA, concentration-response relationships were established for the control and ethanol-treated groups by plotting the peak amplitude of responses as a function of incremental concentrations of GABA. As shown in Fig. 2A, the EC50 of the control group (16 μM; n = 14) was significantly different (P < 0.01, analysis of covariance) from that of the ethanol-treated group (EC50 = 24 μM; n = 7). Thus based on these EC50 values, chronic treatment with ethanol resulted in a rightward shift in the apparent potency of GABA. In addition, the ethanol-fed group showed a reduced maximal current response to GABA (0.751 ± 0.027 nA) compared with the control group (1.027 ± 0.011 nA). This reduction was statistically significant (P < 0.05, unpaired Student’s t-test) and indicated that chronic exposure to ethanol also altered the apparent efficacy of GABA.

Ethanol-GABA interaction

Acute exposure to ethanol has been reported to modulate neuronal responses to GABA (Aguayo 1990; Nestoros 1980; Peoples and Weight 1999). Beyond establishing a decreased sensitivity to GABA itself, a series of experiments examined the interaction between acute ethanol exposure and pyramidal cell responses to GABA and addressed the issue as to whether this interaction was altered in chronic ethanol rats. GABA was applied at a concentration of 10 μM, which approximated the EC25 value (Fig. 2A). Ethanol (10, 25, 50, and 100 mM) or ACSF was continuously delivered between consecutive pressure pulses of GABA. At all concentrations tested, ethanol exposure resulted in a modulation of GABA-induced response...
in both control and chronic ethanol groups. An example of 25 mM ethanol potentiating the GABA response monitored in a pyramidal cell is given in the inset of Fig. 3. Under both control and chronic ethanol conditions, the outcome of the modulation varied, as ethanol potentiated, attenuated, or had no effect on GABA responses in individually tested pyramidal cells. Figure 3 summarizes averaged ethanol-induced changes in GABA response irrespective of the direction of modulation. No clear-cut difference emerged at any of the ethanol concentrations tested \( P > 0.2 \), two-way analysis of variance (ANOVA).

The modulatory effect of acutely applied ethanol was then classified as being either attenuating (GABA-induced current amplitude reduced by >10% of control current), potentiating (GABA-induced current increased by >10% of control current), or having no effect (change in amplitude of GABA-induced current within 10% of control current). The data are summarized in the form of a “scatterplot” (Fig. 4A), which plots the peak amplitude of GABA-induced currents during the control period as a function of that recorded during acute exposure to ethanol. Each point represents data derived from an individual trial. The 45° line predicts no effect of ethanol on the GABA-induced currents. The two lines outlining the shaded “equivalence” zone represent either a 10% increase or decrease in the GABA-induced current response, which was used in this study to determine whether GABA responses observed during exposure to ethanol was potentiated (points lying above the equivalence zone), attenuated (points lying below the equivalence zone), or had no effect (points lying within the equivalence zone). As seen in Fig. 4A, in the chronic ethanol group, 28 out of 52 cases lie above the equivalence zone, indicating potentiation of GABA responses by ethanol. In the control group, only 9 out of 44 cases are found above the equivalence zone. This is consistent with previous in vivo and in vitro studies in which GABA responses displaying sensitivity to potentiation by ethanol were rarely encountered (Carlen et al. 1982; Freund et al. 1993; Harris and Sinclair 1984; Siggins et al. 1987).

Figure 4B represents the binned distribution of the pyramidal cells examined in which ethanol either potentiated, attenuated, or had no effect on GABA responses. An ethanol-induced attenuation of GABA responses was found in control slices (17% of modulatory responses encountered) but never in chronic ethanol slices. Concomitantly, there was a significantly higher percentage of ethanol-induced potentiation in the chronic ethanol group (overall, \( P < 0.001 \), chi-squared analysis). These observations, taken together, indicate that chronic ethanol treatment has the net effect of increasing sensitivity to ethanol-induced potentiation of GABA in piriform cortical pyramidal neurons.

**Modulation by diazepam**

At the cellular level, chronic exposure to ethanol has been reported to produce cross-tolerance to the effects of a variety of GABA\(_A\) receptor modulators, including the benzodiazepines...
The question arose as to whether cross-tolerance to diazepam occurred in the adult rat piriform cortex following chronic treatment with ethanol. In a series of experiments, the identical protocol was used to assess the interaction between acute ethanol and GABA, except that a maximally effective concentration of diazepam (0.5 μM) was substituted for ethanol. As expected of the allosteric modulator, potentiation of GABA-induced currents was seen in all cells. Figure 5A illustrates an example of such a potentiation. Within the population of cells sampled (Fig. 5B), the mean diazepam-induced potentiation of peak GABA-mediated responses in the control (85% ± 15.0; n = 22) and ethanol-fed (182% ± 61.8; n = 20) groups were not significantly different (P = 0.14, unpaired Student’s t-test). Thus, diazepam at the concentration tested in this study did not reveal any cross-tolerance induced by ethanol in piriform pyramidal neurons.

**DISCUSSION**

This study addressed the issue of functional changes associated with GABA<sub>A</sub> receptors in the adult rat piriform cortex following chronic exposure to ethanol. A standard liquid-diet regimen (Lieber and DeCarli 1982) was employed that is a favored model for behavioral, neurochemical, and biochemical studies of alcoholism. The major finding is that the sensitivity of layer II piriform cortical pyramidal cells to GABA is attenuated but that this does not appear to be associated with the development of tolerance to the acute effects of ethanol nor to cross-tolerance insofar as sensitivity to diazepam remained unchanged.

A potentially confounding factor that may affect the inter-
pretation of the data indicating a decrease in sensitivity of piriform cortical pyramidal cells to GABA is weight loss, as chronic treatment with ethanol in the form of a liquid diet, has been reported to have a slight anorexic effect (Lieber and DeCarli 1982, 1989a). The measures taken in this study to pair-feed a group of control rats with an isocaloric liquid diet were designed with this effect of ethanol in mind. Earlier studies have directly addressed this issue by altering the ratio of ethanol to either fat content, minerals, or vitamins in the diet and have reported that the effects of chronic ethanol were independent of the liquid-diet regimen (Lieber and DeCarli 1989b; Lieber et al. 1965). Nonetheless, it is acknowledged that this study could have included additional control groups in which normal levels of food intake and body weight are maintained.

The sensitivity of piriform cortical pyramidal neurons to GABA was significantly reduced after 30 days of exposure to ethanol. Studies employing synaptoneurosomes derived from brain tissue have reported that GABA- or muscimol-stimulated chloride flux following chronic ethanol is decreased, increased, or remains unchanged (Allan and Harris 1987; Buck and Harris 1990; Frye et al. 1991, 1996; Morrow et al. 1988, 1990; Sanna et al. 1993; Tremwel et al. 1994). Such preparations routinely involve the use of large amounts of cerebral cortical or cerebellar tissue. In contrast, the present study is restricted to examining identified pyramidal cells in the piriform cortex. The individually identified neurons in the slice preparation are more likely to retain their physical attributes and are thus studied in a milieu that approximates their environment in situ. This consideration strengthens the conclusion that sensitivity to GABA is attenuated in the piriform cortex following chronic exposure to ethanol.

A priori, the change in sensitivity to GABA observed in this study could be accounted for by either a reduction in GABA$_A$ receptor number, a change in subunit expression, or both. Analysis of concentration-response relationships reveals decreases in both apparent efficacy and potency following chronic ethanol treatment. The observed decrease in efficacy can be accounted for by a reduction in the number of GABA$_A$ receptors, leading to the diminished maximal response of piriform pyramidal neurons to GABA. Prevailing evidence, however, does not uniformly support a chronic ethanol-induced decrease in GABA$_A$ receptor binding or number (Rastogi et al. 1986; Thyagarajan and Ticku 1985). The observed decrease in potency, on the other hand, implies the possible expression of GABA$_A$ receptor isofoms that, when activated, shift the concentration-response curve rightward. This would be consistent with the notion that chronic ethanol treatment may lead to up- or down-regulation of GABA$_A$ receptor subunits. Indeed, in a variety of preparations, chronic exposure to ethanol has been shown to alter the expression of certain GABA$_A$ receptor subunit mRNAs and, in some cases, their encoded proteins (Devau et al. 1997; Kang et al. 1998; Mahmoudi et al. 1997; Matthews et al. 1998; Mhatre and Ticku 1992; Montpied et al. 1991).

While changes in receptor number and expression of subunits are not mutually exclusive, and the results of the present study do not rule out either possibility, the finding of a shift in apparent potency in chronic ethanol-treated rats is highly suggestive that a change in subunit expression underlies at least in part the diminished sensitivity to GABA. Interestingly, Purkinje cells examined in cerebellar slices derived from the same chronic ethanol-treated animals used in this study exhibited attenuated sensitivity to GABA that is associated with a change in apparent efficacy but not potency (DW Sapp and HH Yeh, manuscript in preparation). Ongoing studies are addressing the outstanding issue of profiling the GABA$_A$ receptor subunits expressed in layer II pyramidal neurons of the piriform cortex as well as the cerebellar cortex and how they may be affected by the regimen of chronic ethanol treatment used in this study.

An unexpected finding was the absence of tolerance to modulation by acute exposure to ethanol. The hallmark of tolerance in animals chronically exposed to ethanol is the development of an attenuated or abolished response to acutely applied ethanol, as has been shown to occur for GABA$_A$ receptor-mediated responses in the CNS (Allan and Harris 1987; Mihic et al. 1992; Morrow et al. 1988, 1990). Instead, our data indicate, that on chronic exposure to ethanol, GABA$_A$ receptors expressed in pyramidal neurons of the piriform cortex display increased sensitization to acute ethanol. Considering that tolerance to the effects of acute ethanol could not be demonstrated in this study, it might have been predicted that cross-tolerance would not develop either. Rodents chronically exposed to ethanol exhibit diminished benzodiazepine-induced enhancement of muscimol-stimulated chloride flux (Buck and Harris 1990; Sanna et al. 1993), although no changes in benzodiazepine binding have also been reported (Devau and Morrow 1994; Karabath et al. 1980; Mhatre and Ticku 1989; Rastogi et al. 1986). In the present study, the potentiating effect of diazepam on GABA-induced responses in pyramidal cells was similar in the chronic ethanol-treated and control groups. It should be noted that a single concentration of diazepam (0.5 $\mu$M) was tested which, in a previous study (Sapp and Yeh, manuscript in preparation), was shown to be maximally effective in potentiating GABA responses in cerebellar Purkinje cells. This concentration of diazepam also effectively revealed cross-tolerance to diazepam in cerebellar slices taken from the same ethanol-fed animals. It may be that the detection of tolerance and cross-tolerance can be enhanced by sampling more pyramidal neurons or by extending the chronic ethanol treatment beyond 30 days. Given the data at hand, however, it is proposed that layer II pyramidal neurons of the rat piriform cortex may be more resistant to the development of tolerance to ethanol and cross-tolerance to diazepam following chronic exposure to ethanol. Nonetheless, it is acknowledged that a systematic analysis covering a range of diazepam concentrations will need to be undertaken in future studies. Overall, in comparing layer II pyramidal cells to cerebellar Purkinje cells, an emerging picture is that the effect of chronic ethanol treatment on GABA$_A$ receptor function involves multiple components that become manifested in a brain region- and perhaps even cell type-specific manner.

Tolerance has been proposed to be a protective response on chronic exposure to potentially harmful substances (Littleton 1983). The lack of tolerance to acutely applied ethanol and of cross tolerance to diazepam after 30 days of chronic ethanol treatment suggest that layer II pyramidal neurons of the rat piriform cortex possess GABA$_A$ receptors that are resistant to developing the degree of tolerance seen in other brain regions. The functional implication of this resistance to tolerance remains to be elucidated. However, it is tempting to speculate that this property of piriform cortical pyramidal neurons may
be a factor in contributing to the susceptibility of the piriform cortex to seizures, such as those that can occur on withdrawal from ethanol. In addition, it may play a role in the development of experimentally induced neurodegeneration in animal models of alcoholism as well as of olfactory deficits in human alcoholism.

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